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The Effect of Post-Operative Analgesics on Ovarian Medullary Angiogenesis and Vasculogenesis

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THE EFFECT OF POST-OPERATIVE ANALGESICS ON OVARIAN MEDULLARY ANGIOGENESIS AND VASCULOGENESIS

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ABSTRACT

THE EFFECTS OF POST-OPERATIVE ANALGESICS ON OVARIAN MEDULLARY ANGIOGENSIS AND VASCULOGENESIS

by Shamus M. Brady

A critical factor for successful ovarian transplantation is the expeditious establishment of sufficient blood supply. Recommendations intended to improve recovery, reduce the effects of stress, and decrease the amount of pain for laboratory animals undergoing surgical procedures include post-operative analgesia. The two main types of drugs that are recommended for pain management are opioids and non-steroidal anti-inflammatory drugs (NSAIDs). Buprenorphine, an opioid, and meloxicam, an NSAID, are both widely used and have been shown to affect angiogenesis and vasculogenesis. This study was designed to examine the influence meloxicam and buprenorphine had on new blood vessel formation in the ovarian medullary region of aged female recipient CBA/J mice, transplanted with young ovaries from CBA/J donor females. Medullary vessel analysis was performed by viewing 40 μm thick sections fluorescently labelled with the cell marker CD31/PECAM-1 via confocal microscopy. A multivariate analysis of variance (MANOVA) was performed between treatment groups to analyze how the independent variables of analgesic administration affected multiple dependent variables of deep microvessel quantities. Results demonstrated no significant endothelial microvessel growth or reduction among the meloxicam or buprenorphine-treated mice as compared to saline-treated mice. Results further suggested that neither type of analgesic drugs affected medullary ovarian angiogenesis and vasculogenesis after ovarian transplantation of young ovaries into aged females.

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Introduction

The mammalian ovary performs two major functions: the production of germ cells (oocytes), and synthesis of signaling molecules such as the steroid hormones estrogen and progestins as well as peptide growth factors (Edson, Nagaraja, & Matzuk, 2009). These signal molecules are part of an endocrine axis known as the hypothalamic-pituitary-ovarian axis, responsible for the production of germ cells as well as development of secondary sex characteristics (Edson *et al.,* 2009). The axis originates in the hypothalamus, projects a short distance to the anterior pituitary, and circulates a longer distance to the ovary (Ginther, Dierschke, Walsh, & Del Campo, 1974). The ovarian artery enters the ovary through the hilus or stalk and is initially localized deep into the medullary stroma, the tissue consisting of blood vessels and connective tissue that provides the structural support for the developing follicles (Brown and Russell, 2013). The artery divides into several branches that run within the deep medullary region, separating into many afferent arterioles and capillaries (Kikuta, Macchiarelli, & Murakami, 1991; Courbiere *et al.*, 2009). The medullary afferent arterioles eventually branch at the superficial cortex region to form capillary plexuses (Kikuta *et al*., 1991). The vasculature between the cortical and medullary regions of the ovary differs in structure and function. The arteries in the medulla are larger in size and highly tortuous, and the vasculature within the cortex includes arterioles and primary capillary beds (Kikuta *et al*., 1991). The cortical capillary beds provide nutrients and oxygen, while facilitating CO_2 and waste removal from follicles ranging from primordial to developing

primary and secondary follicles that have the potential to develop into a mature ovum (Fortune, Cushman, Wahl, & Kito, 2000; Brown and Russell, 2013).

Blood vessels are initially formed in the embryo via angiogenesis and vasculogenesis (Bussolino, Mantovani, & Persico, 1997). Angiogenesis is the development of new vessels from preexisting blood vessels while vasculogenesis is the arrangement of new blood vessels from angioblasts, primitive mesodermal cells of the primary germ layers committed to differentiate into endothelial cells (Campochiaro, 2000; Risau and Flamme, 1995). Angioblasts arise from the mesodermal layer during embryogenesis in a process called gastrulation (Coffin and Poole, 1988). During the early stages of vasculogenesis, mesodermal angioblasts tightly aggregate near the endodermal layer to form a blood band of cells formally known as blood islands (Sabin, 1920; Vokes and Krieg, 2002; Ferkowicz and Yoder, 2005). The blood band contains a collection of outer angioblasts and an inner core of hematopoietic stem cells, called hemangioblasts (Murray, 1932; Ferkowicz and Yoder, 2005). The hemangioblasts differentiate into angioblasts and then further differentiate into endothelial cells forming the lumen and basal lamina of the capillary plexus (Noden, 1990). By late neural plate stage, the blood band is 8-15 cells in diameter as it circumferentially wraps around the proximal yolk sac with a band thickness of 2-4 cells (Ferkowicz and Yoder, 2005). The endothelial cells begin to remodel the blood band into smaller channels with blood cell filled capillary beds, connected to the primitive capillary plexus (Sabin, 1920; Risau and Flame, 1995; Ferkowicz and Yoder, 2005). A small portion of blood vessels formed during embryonic development remain until the adult stage due to primitive vascular

plexus regression, thought to be the result of lack of blood perfusion (Risau and Flamme, 1995). The development of a vasculature during later embryonic and adult stages is produced via angiogenesis (Risau and Flamme, 1995).

Angiogenesis occurs in the yolk sac as well as the developing embryo by sprouting from pre-existing vessels and forming a capillary plexus (Risau, 1997). The angiogenic process is necessary for normal ovarian function and is stimulated by hypoxia, low levels of oxygen, induced by ischemia or lack of adequate blood supply to ovarian endothelial cells (Bamberger, 2002; Shweiki, Itin, Neufeld, Gitaygoren, & Keshet, 1993). Endothelial cells within the ovary respond within 1-2 hr of hypoxia with increased expression of vascular epithelial growth factor (VEGF), an important angiogenic signal molecule (Folkman and Klagsbrun, 1987; Folkman, 2003). Once VEGF is released and binds to the vascular epithelial growth factor receptor (VEGFR) located on adjacent endothelial cells, it has multiple angiogenic roles including inducing endothelial cell division, promoting endothelial cells to form vessels by clustering on adjacent capillaries, aiding in vascular leakage, and increasing hydraulic conductivity of isolated microvessels (Schieki *et al*., 1993; Risau, 1997; Ferrara, Gerber, & LeCouter, 2003). Additionally, VEGF promotes the release of matrix metalloproteinase-9 (MMP-9), an important extracellular matrix remodeling molecule from endothelial cells which causes the degradation of extracellular matrix allowing space for new blood vessel formation (Ferrara *et al*., 2003). Finally, the newly formed endothelial cells adhere and create tubes and loops that work as functional vascular units (Ferrera *et al.*, 2003; Folkman and Klagsbrun, 1987; Risau, 1997).

Ovarian blood vessel formation via vasculogenesis and angiogenesis is not limited to embryological phases, but occurs throughout mammalian life (Asahara *et al.,* 1999). During adult vasculogenesis, angioblasts from the mesodermal layer are no longer involved. Instead, endothelial progenitor cells from the bone marrow circulate through the blood, normally in small quantities, and contribute to new blood vasculature (Asahara *et al*., 1999). The bone marrow-derived cells are thought to be recruited by pro-angiogenic factors, especially VEGF and stromal derived-factor 1 (SDF-1) (De Palma, & Naldini, 2006; Li *et al.,* 2006). The circulating endothelial progenitor cells (CEPCs) express both VEGFR and chemokine receptors (CXCR4) on their cell surfaces, binding to VEGF and SDF-1, respectively (De Palma *et al*., 2006). The binding of VEGF and SDF-1 leads CEPCs to the area of ischemia where they differentiate into endothelial cells and form the central region of new blood vessels (Asahara *et al*., 1999). The total contribution of CEPCs appears to be limited to the area of new blood vessel formation and does not contribute to fully established adult vasculature (Crosby *et al*., 2000). Furthermore, angiogenesis within the ovary contributes to the remodeling of the early blood vasculature previously formed in embryos (Brown and Russell, 2013). The process takes place concurrently with folliculogenesis, the development of follicles in the ovary, and leutinization, the formation of the corpus luteum after ovulation (Brown and Russell, 2013; Mosby, 2009). Ovarian angiogenesis is vital for increasing delivery of the gonadotropins, luteinizing hormone, follicular stimulating hormone, growth factors, oxygen, lipids, and steroid precursors required for folliculogenesis (Brown and Russell,

2013). Folliculogenesis and luteinization occur in the cortical region of the ovary, therefore angiogenesis occurs largely in that area (Fortune *et al*., 1999).

Neovascularization via vasculogenesis and angiogenesis returns transplanted tissue to a homeostatic environment, reestablishing appropriate blood supply, and is proven to be critical for transplantation success (Gourishankar and Halloran, 2002; Marzouk, Lawen, Alwayn, & Kiberd, 2013). A major obstacle in the survival of an ovarian transplant is vascular failure, which leads to tissue necrosis (Israely *et al*., 2003). Proper blood vascular connection post-transplantation provides important nutrients like water, salts, sugars, fats, and proteins, and removes metabolic waste (Cohen, 1945). Research utilizing oxygen consumption and terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL), an apoptosis assay, determined that ovarian cortical sections incubated in ischemic minimal essential medium can survive for only 24 hr without proper blood supply before experiencing significant apoptosis (Kim *et al*., 2004). Additional research examining vascularization of xenograft rat ovaries determined ischemia causes medullary tissue damage 24-48 hr after grafting (Israely *et al*., 2003). Therefore, ovarian transplant survival improves when conducted in conditions that support vascular maintenance (Israely *et al*., 2003).

Recommendations given by Institutional Animal Care and Use Committees (IACUC) as well as veterinary medical practitioners are intended to improve recovery, reduce the effects of stress, and decrease the amount of pain by providing post-operative analgesia for laboratory animals undergoing surgical procedures (Kohn *et al.,* 2007). The two main classes of drugs that are recommended for pain management are opioid and

non-steroidal anti-inflammatory drugs (NSAIDs). Within the two analgesic classes, meloxicam and buprenorphine are recommended for administration either alone or combined depending on the level of pain the host may experience (Kohn *et al.,* 2007). The most commonly used analgesic for post-operative care in mice is buprenorphine, which is used for moderate to severe pain and has a prolonged duration of analgesic action (Gargiulo *et al*., 2012). Meloxicam has the advantage of being more easily consumed orally and is less likely to result in gastrointestinal disturbances (Gargiulo *et al*., 2012; Blaha and Leon, 2008). While it is vital to ensure proper comfort and pain management for laboratory animals undergoing organ transplantation, it is also important to evaluate whether either class of drugs could potentially affect vasculogenesis and angiogenesis.

Buprenorphine is an opioid used as an analgesic in both humans and animals (Cowan, Doxey, & Harry, 1977). Buprenorphine is a semisynthetic opiate from the precursor thebaine and is specific for the OP3/μ receptor, an opioid receptor that has been shown to have anti-nociceptive effects, as well as exhibit slow receptor kinetics and sustained activation (Garrett and Chandran, 1990; Lutfy *et al*., 2003). Activating the OP3/μ receptor also causes sedation, a drop in blood pressure, and decreased GI motility (Johnson, Fudala, & Payne, 2005). Due to the slow receptor kinetics, buprenorphine is considered to be a long-acting analgesic with a low physical dependence (Cowan *et al*., 1977; Johnson *et al*., 2005).

Research examining the effects of opiate/opioid drugs on angiogenesis has been conflicting. Morphine, a commonly used opiate analgesic, has been shown to stimulate

microvascular endothelial cell proliferation and angiogenesis in a murine MCF-7 cell breast tumor xenograft model *in vivo* at a dose of 0.714 mg/kg for 15 days followed by a dose of 1.43 mg/kg for 23 days (Gupta *et al*., 2002). Furthermore, research conducted *in vitro* using a murine matrigel model with 10 μM morphine and 10 μM morphine/10 μM naloxone (an opioid antagonist), for 10 days showed similar results (Gupta *et al*., 2002). This research found that an increase in angiogenesis was achieved by activating mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) phosphorylation and is dependent on nitric oxide (NO) in microvascular endothelial cells (Gupta *et al*., 2002). Morphine and VEGF have been shown to increase angiogenesis through a similar mechanism (Gupta *et al*., 2002). In a separate study, morphine was topically applied to rats that had an open ischemic wound, and after one week morphine increased angiogenesis by 45-87% compared to the phosphate buffered saline control group (Poonawala, Levay-Young, Hebbel, & Gupta, 2005). The increased angiogenesis and wound healing was explained by up-regulation of VEGF, inducible nitric oxide synthase, and the VEGF receptor *flk-1* in the wounds (Poonawala *et al*., 2005). In a conflicting study, mouse heart microvascular endothelial cells treated with morphine-sulfate at a dose of 100 ng/mL and then incubated for 24 hr in hypoxic conditions, showed markedly reduced VEGF expression and reduced *Vegf* mRNA (Balasubramanian *et al*., 2001). In a similar study, nude mice treated subcutaneously with Lewis lung carcinoma cells (LLCs) were given morphine, control, or a morphine/naltrexone combination at a dose of 200-400 ng/mL for 7 days, followed by an additional dose of 20 mg/kg/day for days 7-14, followed by 30 mg/kg/day for days 15-21 (Koodie,

Ramakrishnan, & Roy, 2010). These investigators found that morphine reduced vessel density, vessel branching, and vessel length. Additionally, LLCs were administered 1.0 μmol/L morphine sulfate and grown on tissue-cultured treated dishes and examined for VEGF-A expression via real-time PCR (Koodie *et al*., 2010). This study found a significant reduction in tumor cell-induced angiogenesis, VEGF-A expression, vessel density, vessel branching, and vessel length with cultured LLCs and mice treated with morphine sulfate (Koodie *et al*., 2010).

In addition to opioid derivatives, meloxicam is an NSAID that binds specifically to cyclo-oxygenase-2 enzymes (COX-2). COX-2 plays an important role in inflammation by stimulating prostaglandin synthesis (Vane, 1994; Willoughby, Moore, & Colville-Nash, 2000). Under the normal inflammatory pathway, arachidonic acid is converted to prostaglandin-G2 (PGG2) by COX-1 or COX-2 (Vane, 1994). PGG2 is converted to prostaglandin-H2 (PGH2) by COX-1 or COX-2 (Hamberg, Svensson, & Samuelsson, 1975). PGH2 can undergo five separate conversion pathways that can yield several prostanoids (Vane, 1994). Prostanoids function as autocrine and paracrine factors that mediate the inflammatory response (Ricciotti and FitzGerald, 2011). Meloxicam and other NSAIDs irreversibly acetylate the COX-2 enzyme (Vane, 1994). Research has shown that meloxicam, when administered to Wistar rat gingival margins at a dose of 3 mg/kg/day, intraperitoneally, for 3, 7, 14, and 30 days can serve as an inhibitor to angiogenesis by reducing mRNA expression of VEGF, a key angiogenic and vasculogenic growth factor (Oliveira *et al*., 2008). In a separate study, pellets containing the pro-angiogenic factor basic Fibroblast Growth Factor (bFGF) were implanted into the

cornea of rats (Leahy *et al*., 2002). Once the pellets were implanted, meloxicam at a dose of 30 mg/kg/day or saline was administered by gavage twice per day for four days. After four days, the rat corneas showed a significant (78.6%) reduction in angiogenesis due to inhibition of the COX-2 enzyme (Leahy *et al*., 2002). The previous two studies indicate the potential for meloxicam and other COX-2 inhibitors to reduce angiogenesis by decreasing the production of prostaglandins and the expression of VEGF (Oliveira *et al*. 2008; Leahy *et al*., 2002).

Confocal microscopy is widely used for visualization of blood vessels (McDonald and Choyke, 2003). It uses a focused laser beam to illuminate a specific focal plane in tissue (Webb, 1996). The light that is reflected from that focal point enters through a pinhole on the microscope and acts as a size-spatial filter that limits the amount of light that eventually returns to the detector (Aghassi, Anderson, & González, 2000). Confocal microscopy has the advantage of increased contrast, especially on thick specimen microscopic images (McDonald and Choyke, 2003). The advantage of focusing on such a small focal plane is the reduction in scattering of out-of-plane light as well as improved image contrast and resolution compared to wide field microscopy (Webb, 1996).

Confocal microscopy can be used for either light or fluorescent microscopy; however, it is more commonly used for fluorescent imaging to detect specific cellular components (McDonald and Choyke, 2003). Confocal microscopy is often utilized to study angiogenesis and vasculogenesis by fluorescently labeling specific cell markers that target neovascularization. An important antigen, vital for vascular cell adhesion, is the cluster of differentiation-31/platelet endothelial cell adhesion molecule (CD31/PECAM-

1) (Albelda, Muller, Buck, & Newman, 1991). This particular protein is approximately 100 amino acids in length and is responsible for the hydrophobic core in protein-protein interactions that connects endothelial cells to adjacent endothelial cells during neovascularization (DeLisser, Baldwin, & Albelda, 1997; Russell, Breed, & Barton, 1992). This specific endothelial antigen has been used to detect postnatal vasculogenesis in bone marrow-derived cells, angiogenesis in quail and chicken endothelial cells, and microvessel density in breast cancer patients (Asahara, 1999; Kim *et al.,* 2009; Drake, Brandt, Trusk, & Little, 1997; Horak *et al.,* 1992).

Two powerful types of pain relieving medicines, buprenorphine, an opioid, and meloxicam, an NSAID, are both widely used and have both shown to affect angiogenesis and vasculogenesis. The aim of this study was to examine the impact meloxicam and buprenorphine had on ovarian medullary blood vessel formation of aged female recipient CBA/J mice transplanted with young ovaries from donor CBA/J mice. Medullary vessel analysis was performed by viewing tissue sections fluorescently labelled with specific cell marker CD31/PECAM-1 on a confocal microscope. An analysis was performed between treatment groups to analyze how the independent variables of analgesic administration affected medullary angiogenesis and vasculogenesis by measuring multiple dependent variables of deep microvessel quantities.

Methods and Materials

Ethics Statement

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. All

experimental procedures were approved by the Institutional Animal Care and Use Committee at San José State University (protocol 959). Surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Animals

Female recipient CBA/J strain mice (Jackson Laboratory, Sacramento, CA) were purchased at seven weeks of age and housed at San José State University. Their housing conditions included: five mice per 29 x 22 x 14 cm cage, a daily photoperiod of 12L:12D, and *ad libitum* access to food (Purina Mouse Chow 5008: 23.5% protein, 6.5% fat, Purina Mills, St. Louis, MO) and water (Deionized). Age matched male mice were housed in adjacent cages to promote estrous cycling (Whitten, 1956). Two weeks prior to surgery, donor mice of the same line were purchased (Jackson Laboratory, Sacramento, CA) at six weeks of age and housed under the same living conditions.

Ovarian Transplantation and Administration of Treatment

Following surgical procedures previously described by Le, Bonachea, and Cargill (2014), female recipient mice, aged to 11 months, were anesthetized with an intraperitoneal injection of sodium pentobarbital (0.65 mg/kg of body weight dose, Lundbeck Inc., Deerfield, IL) and their ovaries were surgically removed and immediately replaced with 2-month old ovaries from CBA/J donor mice (Jackson Laboratory, Sacramento, CA) into the ovarian bursae. The ovarian bursa and the abdominal wall were closed with 7-0 Ethilon nylon filament and 5-0 Ethicon chromic gut (Ethicon, San Angelo, TX), respectively. Wound clips (Becton Dickinson and Company, Sparks, MD) were used to close and secure the skin and were removed one week post-surgery.

Prior to surgery, recipient mice were randomly assigned to one of three treatment groups, meloxicam (Boehringer Ingelheim, St. Joseph, MO), buprenorphine (Reckitt Benckiser, Hull, England), or 0.9% saline (Vedco, Inc., St. Joseph, MO) (no analgesic). One of the 9 saline mice died during surgery, likely due to an overdose of anesthetic. A second saline injected mouse died approximately 1 month post-surgery, most likely due to seizures known to affect this line (Fuller, & Sjursen Jr, 1967). This brought the total saline injected to a sample size of seven. The mice in the meloxicam treatment group animals were administered intraperitoneal doses of 5 mg/kg meloxicam. The buprenorphine treatment group animals were administered intraperitoneal doses of 0.05 mg/kg. The control treatment group animals were administered 0.9% saline in similar volumes to the two drug treatment groups. Current veterinary guidelines and recommendations as well as previous studies were followed to determine the proper doses of analgesics (Mason, Cargill, Anderson, & Carey, 2009; Flecknell, 2009; Plumb, 2002). The first dose of each treatment was given after ovary allotransplantation and before anesthetic recovery. Doses of meloxicam, buprenorphine, and saline were administered every 12 hr for 48 hr post-operatively.

Fixation and Preservation of Ovaries

For non-biased double-blind analysis all female recipient mice were randomly assigned new identification numbers and sacrificed by cervical dislocation between 73-78 days post-transplantation. One ovary from each mouse was placed in a 5 ml glass vial (Wheaton Science Products, Millville, NJ) containing 4 mL of immunohistochemistry (IHC) zinc fixative (BD Biosciences, San Diego, CA). After incubation at 4º C for 24 hr,

tissue forceps were used to transfer the ovaries to a new vial containing RNase-free phosphate buffered saline (PBS) (Boston Bioproducts, Ashland, MA) and secured with parafilm. The ovaries were placed in a cardboard container and stored at 4º C. The containers were packed on dry ice in a Styrofoam cooler for shipping. They were shipped to IHC World LLC (Ellicott City, MD) for surface CD31 immunohistochemistry staining and 3,3'-diaminobenzidine (DAB) chromogen staining.

Surface CD31 Immunohistochemistry Procedure

Upon receipt of samples the 5 ml labeled vials were removed from 4º C and ovaries were transferred to labeled 1.5 ml microcentrifuge tubes containing room temperature RNase-free PBS (Fisher Scientific, Fair Lawn, NJ). The ovaries were first washed in PBS-Tween20 (Fisher Scientific, Fair Lawn, NJ) for 30 min followed by incubation in rabbit anti-mouse CD31 primary antibody (Thermo Scientific Pierce, Rockford, IL) diluted 1:100 using IHC-Tek Antibody Diluent (IHC World, Ellicott City, MD) on a shaker at room temperature for 16 hr. Directly after incubation, the ovaries were washed in PBS-Tween20 three times for 30 min each and incubated on a shaker in 3% Peroxidase Blocking Solution (Vector Laboratories, Burlingame, CA) for 20 min at room temperature. This was followed by an additional three washes in PBS-Tween20 for 30 min each, the ovaries were incubated in biotinylated goat anti-rabbit secondary antibody (Thermo Scientific Pierce, Rockford, IL) diluted 1:500 using IHC-Tek Antibody Diluent on a shaker at room temperature for 16 hr. Following this incubation, the ovaries were washed in PBS-Tween20 three times for 1 hr each and incubated on a shaker in a 1:500 dilution of HRP-Streptavidin (Biolegend, San Diego, CA) for 16 hr at room

temperature. The ovaries were then washed in PBS-Tween20 three times for 1 hr each and incubated in a 1:4 dilution of 3,3'-diaminobenzidine (DAB) chromogen (Fisher Scientific, Fair Lawn, NJ) for 10-15 min at room temperature, removing the ovaries when a uniform dark brown color had been achieved. Once the ovaries turned the proper color, they were rinsed with three washes of PBS-Tween20 at 10 min each and placed in new labeled 1.5 ml microcentrifuge tubes containing 1 ml of 50% glycerol (Hampton Research, Aliso Viejo, CA) in PBS. The ovaries were incubated in the 50% glycerol/PBS solution for 10-20 min until the ovary sank to the bottom of the tube. The ovaries were then removed and placed in a 70% glycerol in PBS solution and the labeled tubes were stored at 4º C. The ovaries were shipped from IHC World LLC (Ellicott City, MD) overnight to San José State University at 4º C.

Paraffin Embedding

Upon receipt the ovaries were transferred from the 5 mL labeled vials at 4° C, using fine tissue forceps, to new 1.5 mL sterile microcentrifuge tubes containing room temperature RNase-free PBS. The ovaries were dehydrated by transfer to 1.5 mL microcentrifuge tubes (Fisher Scientific, Fair Lawn, NJ) containing 70% ethanol (Histo-chem, Jefferson, AR) and placed on a mixer at room temperature for 60 min. This step was repeated once with 95% ethanol and twice with 100% ethanol. Finally the ovaries were incubated three times with 100% xylene (Sigma-Aldrich, St. Louis, MO) for 1.5 hr each at room temperature. After they were removed from the xylene, they were placed into a 200 mL beaker containing molten (60º C) paraffin wax (Paraplast Plus, Sigma-Aldrich) for 2 hr. The ovaries were transferred, using warm sterile forceps, to the well of

an embedding cassette (Fisher Scientific, Fair Lawn, NJ). Molten paraffin wax was poured into the well of the cassette over the ovary. The ovary and cassette were stored at room temperature for 6 hr. After drying, the paraffin embedded ovaries were sectioned with a Leica Histoslide 2000 Sliding Microtome (Leica Microsystem, Buffalo Grove, IL). Surface ovary sections of 18 μm were used for another experiment. Depending on the size of the ovary, surface removal ranged from 108 μm for small ovaries to 378 μm for large ovaries. Medullary sections of 40 μm each were mounted on TruBond 380 microscope slides (TruBond, Woodstock, MD). The slides were air dried for 30 min followed by baking at 45º C for 16 hr in a Precision Scientific 25EM oven (Thermo Electron Corporation, Marietta, Ohio). Rehydration of the ovary sections was performed by three separate washes at room temperature with 100% xylene for 5 min each. The slides were immersed one time for 5 min in room temperature 100% ethanol. This step was repeated with 95% ethanol, 70% ethanol, and 50% ethanol, respectively. The slides were washed twice with RNase-free PBS for 10 min each at room temperature. The slides were dried, and any excess PBS was blotted using Kimwipes (Kimberly-Clark, Irving, TX).

Immunofluorescence

Alexa Fluor fluorescent labeled polyclonal antibody (Abcam) staining was performed using the manufacturer protocol (BD Biosciences Franklin Lakes, NJ). Briefly, slides were submerged in rabbit polyclonal CD31/PECAM-1 primary antibodies (1:50 dilution; Abcam) overnight at 4º C. The slides were washed two times with room temperature PBS for 10 min each. Excess PBS was blotted from the slide using

Kimwipes. The slides were incubated for 60 min at room temperature submerged in goat anti-rabbit polyclonal secondary antibodies labeled with Alexa Fluor 488 (1:100 dilution; Abcam). The slides were washed twice with PBS for 10 min each. Excess PBS was blotted from the slide using Kimwipes. One drop of VECTASHIELD mounting medium with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) was placed on each ovary section, which was then topped with a coverslip.

Deep Microvessel Imaging

Whole ovary section images were acquired using a Zeiss LSM 700, Axio Imager 2 confocal microscope with the 20x/0.8 M27 objective. The microscope detection wavelength ranges from 493-800 nm with a pinhole size of 27.67 μm. The Alexa Fluor 488 fluorescent label emitted light that was captured at approximately 519 nm. The images were taken in the tile format where the microscope performs a sweeping scan on the section taking several images during the sweep. These images were combined to form one complete image. The combined image dimensions were 1.92 mm X 1.92 mm. A representational image of all three subject groups, at a depth of 328 ± 4 μm, shows the intensely illuminated green areas of blood vasculature (Figure 1). The images were analyzed using ZEN software (Zeiss, Jena, Germany). The ZEN software circle, line, and spine contour graphic tools were used to measure the deep microvessel quantities (Figure 1). Individual vessels were measured and their data transferred to an excel spreadsheet (Microsoft Office, Microsoft Corporation, Redmond, WA).

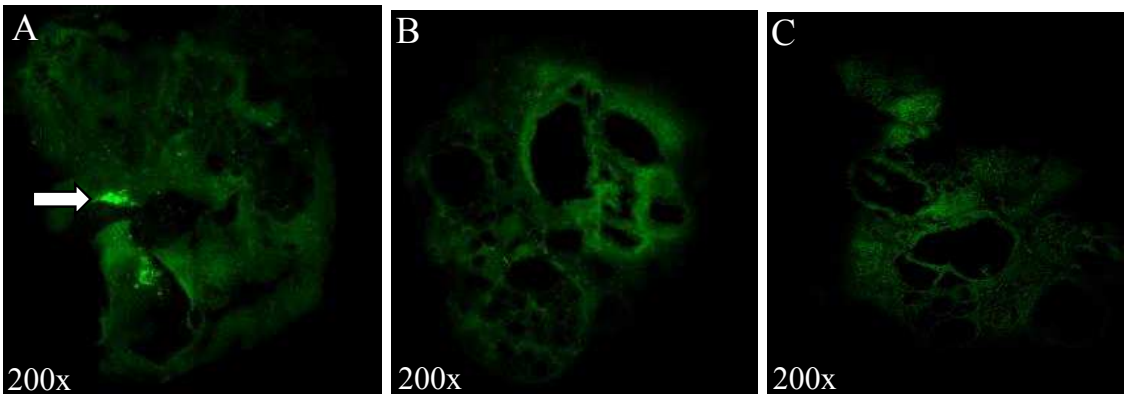

Figure 1. Treatment group Alexa Fluor 488-labeled 40 µm ovary sections. All sections are from a depth of 328 µm ± 4 µm. Images are at 200x total magnification. Intensely illuminated green areas indicated blood vasculature (white arrow). (A) ovarian section from saline treatment group mouse, (B) ovarian section from meloxicam treatment mouse, (C) ovarian section from buprenorphine treatment group mouse.

Deep Microvessel Quantification

The mean number of vessels, diameter of vessels, length of longitudinal vessels, number of vessel branches, the length of each branch, number of vessel junctions, surface area of the total ovary section, number of branches on a longitudinal vessel, and number of junctions per longitudinal vessel were calculated by averaging all measured values within each treatment group.

Statistical Analysis

The treatment group data was transferred to the SPSS (Version 22.0) computer software package (IBM, Armonk, NY). A multivariate analysis of variance (MANOVA) was performed between treatment groups to analyze how the independent variables of analgesic administration affected multiple dependent variables of deep microvessel quantities as listed in the previous section. The MANOVA statistical test is a more suitable test for comparing multiple dependent variables that are moderately related

(Cole, Maxwell, Arvey, & Salas, 1993). Data are presented as means ± standard deviations.

Results

The medullary ovarian vasculature was examined by counting each section of tissue for the total number of blood vessels, number of vessels per cross sectional area, the number of vessel branches, number of junctions, and the relationship of each parameter to overall mean vessel count of all three treatment groups was analyzed. Branches are defined as an area where two blood vessels intersect and junctions are defined as an area where three or more vessels intersect (Su, Sun, & Pham, 2012) (Figure 2).

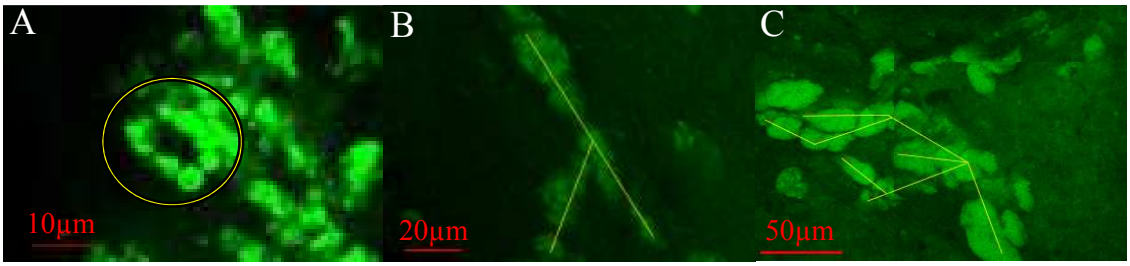

Figure 2. Representative images of meloxicam-treated murine sections. All sections are 40 µm and the images are at 200x total magnification. A) Vessel diameter with yellow circle indicating the location of a vessel cross section, B) Vessel branching with yellow lines represent blood vessel tracing, C) Vessel junction with yellow lines representing blood vessel and junctions where three or more vessels combine.

A summary of the ovarian medullary blood vessel data for all three treatment groups is shown in Table 1. None of the medullary blood vessel parameters measured in this experiment exhibited a significant difference when compared between treatment groups. The average number of blood vessels per section for saline was 20.05 ± 5.15,

meloxicam was 24.31 ± 7.15, and buprenorphine was 22.73 ± 8.22 (p = .501). The mean vessel density per section for saline was 20.69 ± 11.61 vessel/cm^2, meloxicam was 23.99 ± 10.33 vessel/cm^2, and buprenorphine was 25.67 ± 16.73 vessel/cm^2 (p = .759). The mean number of branches in saline was 8.49 ± 3.87, meloxicam was 9.77 ± 2.32, and buprenorphine was 7.77 ± 3.83 (p = .454). The mean number of junctions in saline was 2.67 ± 1.62, meloxicam was 3.56 ± 1.67, and buprenorphine was 2.91 ± 1.51 (p = .512). The density of blood vessel branching for saline was 0.412 ± 0.109 branches/vessel, meloxicam was 0.430 ± 0.120 branches/vessel, and buprenorphine was 0.333 ± .0902 branches/vessel (p = .150). The mean number of junctions per vessel for saline was 0.132 ± 0.0624, meloxicam was 0.152 ± 0.0679, and buprenorphine was 0.134 ± 0.0624 (p = .772). The mean vessel diameter measured for saline was 12.05 ± 1.44 µm, meloxicam was 12.12 ± 0.751 µm, and buprenorphine was 11.31 ± 2.18 µm (p = .500). The mean vessel length for saline was 22.45 µm ± 7.30, meloxicam was 25.52 ± 9.93 µm, and buprenorphine was 21.53 ± 4.98 µm (p = .530). The mean branch length was measured for each treatment group, and the value for saline was 19.30 ± 6.08 µm, meloxicam was 14.88 ± 5.60 µm, and buprenorphine was 16.10 ± 4.00 µm (p = .253).

Table 1.

Ovarian medullary vasculature quantities

Ovarian Medullary Measured Parameters	Analgesic Treatment						
	Saline		Meloxicam		Buprenorphine		
	M	*SD*	*M*	*SD*	*M*	*SD*	*p*
Vessel Count	20.05	5.15	24.32	7.15	22.72	8.23	.501
Vessel Density	20.69	11.60	23.99	10.33	25.67	16.73	.759
Vessel Branching	8.49	3.87	9.77	2.32	7.76	3.83	.454
Vessel Junctions	2.68	1.63	3.57	1.67	2.91	1.51	.512
Branching Density	0.412	0.109	0.430	0.120	0.333	0.0902	.150
Vessel Junctions/Vessel	0.132	0.0624	0.152	0.0679	0.134	0.0626	.772
Vessel Diameter (μm)	12.05	1.44	12.12	0.75	11.31	2.18	.500
Vessel Length (μm)	22.45	7.29	25.52	9.93	21.53	4.98	.530
Branch Length (μm)	19.29	6.08	14.88	5.57	16.09	4.00	.253

Note. Mean, standard deviation, and *p*-values for mean vessel count, vessel density, branching, junctions, branching per unit area, junctions per blood vessel, vessel diameter, length, and branch length of medullary ovary sections from saline ($n = 7$), meloxicam ($n = 9$), and buprenorphine ($n = 9$) treated females. Each mean represents the average value for all ovarian sections within the treatment group. Saline ($n = 7$), meloxicam ($n = 9$), and buprenorphine ($n = 9$) treatment groups exhibited no differences in all variables measured.

Discussion

A vital factor for ovarian transplantation success is the immediate establishment of an ample blood supply, which is necessary for the survival of the ovarian follicles and prevention of necrosis and apoptosis (Weissman *et al*., 1999; Eklund, Bry, & Alitalo, 2013; Starlz, Kaupp, Brock, Lazarus, & Johnson, 1960; Brodie, 1903). Extensive revascularization has been shown in juvenile rat ovaries transplanted to an ectopic site within 48 hr (Dissen, Lara, Fahrenbach, Costa, & Ojeda, 1994). Before revascularization is established, transplants are susceptible to ischemia-reperfusion injury which causes a 30-70% reduction in graft size due to tissue necrosis (Kim, Soules, & Battaglia, 2002).

After ovarian transplantation in mice there is extensive medullary necrotic damage after 24 hr, with necrosis in almost all the medullary tissue and most of the cortical region (Israely *et al*., 2003). However, transplanted ovaries are able to recover from exhibiting a poor quality medullary area three days after transplant to resembling a normal ovary six days post-transplant (Israely *et al*., 2003). An even greater improvement in graft recovery and revascularization was realized by injecting endothelial progenitor cells (EPC) and VEGF into transplantation sites and inducing adult vasculogenesis (Cha *et al*., 2014). Therefore, under normal and EPC/VEGF injected transplantation conditions, the medullary region is capable of recovering from necrosis due to ischemia and is able to fully function.

Since tissue health is dependent on the duration of ischemia, any inhibition of blood vessel formation could be detrimental to transplantation success (Kim *et al*., 2004; Starlz *et al.*, 1960). Analgesics can alter angiogenesis and vasculogenesis, and analgesics can have a negative impact on ovarian transplantation. In order for the ovarian medulla to remain viable, oxygenated blood must be returned within 24-48 hr (Israely *et al*., 2003). Any factor that slows the delivery of oxygenated blood to the medulla can result in tissue necrosis. In the present study, the vasculature was severed during surgery, and as a result, meloxicam and buprenorphine were not carried to the ovary through the blood by means of the ovarian artery branching and entering the medulla and eventually entering the cortex of the ovary (Kikuta *et al*., 1991). On the contrary, meloxicam and buprenorphine would need to diffuse through the outer cortex before reaching the deeper medulla of the transplanted ovary. Since the analgesic protocol in this current research administered the

treatment every 12 hr for the first 48 hr after surgery, the treatment analgesics would have needed to penetrate, via diffusion, deep into the medulla during that time to potentially influence angiogenesis (Le *et al*., 2014; Mason *et al*., 2009). It is unlikely for meloxicam (MW = 351 g/mol) and buprenorphine (MW = 467 g/mol) to diffuse from the outer cortical regions to the deeper medullary regions, ranging from 200-580 μm in depth, during the 48 hr of periodic administration.

Meloxicam and buprenorphine have been shown to effect neovascularization (D'Albora, Anesetti, Lombide, Dees, & Ojeda, 2002; Gupta *et al*., 2002). Meloxicam is an NSAID that has been shown to reduce angiogenesis by specifically inhibiting COX-2 and decreasing vessel sensitivity to bradykinins and histamines, which reverses vasodilation in the inflammatory response, or by reducing the expression of VEGF (Katzung, Masters, & Trevor, 2009; Oliveira *et al*., 2008). Buprenorphine's targets are opioid receptors, specifically OP3 on neurons in the central and peripheral nervous system, neuroendocrine, immune, and ectodermal cells (Nalini, Smith, & Manchikanti, 2011). During development the ovaries are equipped with a network of intrinsic neurons, and the medullary region contains neurons most often observed close to blood vessels (D'Albora *et al*., 2002). The function of these neurons is still being investigated. They have been shown to produce nitric oxide (NO) and respond to the neurotransmitters neuropeptide-Y and catecholamines (D'Albora *et al*., 2002). The roles of neurons responsive to neurotransmitters and catecholamines are thought to be regulative on ovarian physiology, and the NO produced by ovarian neurons has been shown to inhibit steroidogenesis through autocrine signaling and promote local vasodilation by paracrine

signaling (D'Albora *et al*., 2002). Previous research examining the expression of peripheral opioid receptors demonstrated the OP3 receptors are expressed on neurons in the ovaries of rats (Wittert, Hope, & Pyle, 1996) This indicates that the specific receptor for buprenorphine is present in ovarian tissue. The physiological role of these receptors in the medulla of the ovary is still being investigated, however, the results of this study did not find any evidence of a regulatory role in vessel formation via angiogenesis or vasculogenesis post-ovarian transplantation under the current administration protocol.

The previously reported literature on the angiogenic effects of meloxicam and buprenorphine have been conflicting. Gupta and colleagues (2002) showed that morphine promoted endothelial microvessel growth. Their study examined human breast cancer xenografts, by using cell cultures and mammary fat pad and determined that morphine activates the MAPK/ERK and NO-dependent pathways to promote angiogenesis (Gupta *et al*., 2002). In a separate study, Balasubramanian *et al*. (2001) found that morphine-sulfate markedly reduces the expression of VEGF and inhibited vascular growth. Their research used cell cultured cardiomyocytes and examined VEGF mRNA levels to show how morphine-sulfate inhibited angiogenesis and vasculogenesis (Balasubramanian *et al.,* 2001). Meloxicam administered to Wistar rat gingival margins showed a decrease in angiogenesis by reducing mRNA expression (Oliveira *et al*., 2008). The finding of the previously mentioned studies are inconsistent and conflict with this current research. The results in the current study did not indicate medullary endothelial microvessel growth nor reduction in the meloxicam or buprenorphine treated mice. A possible explanation for these differences may be due to the fact that different tissue

types were used in each study. Gupta *et al*., Balasubramanian *et al*., and Oliveira *et al*. all examined superficial tissue while this study measured deep medullary vasculature. Higher percentages of angiogenesis is expected in superficial regions like the cortex versus deeper medullary regions (Cursiefen, Kuchle, & Naumann, 1998; Kang *et al*., 2001). The cortical region of the ovary exhibits greater angiogenesis and is closer to any potential blood supply from the host. Consequently, the analgesic treatments administered would have to penetrate significantly deeper into the ovarian tissue to exhibit a significant angiogenic influence on the deep medullary region.

A factor limiting the concentration of analgesics reaching the ovarian medulla is the elimination pharmacokinetics of meloxicam and buprenorphine in tissue. Meloxicam, when given to mice intravenously at 10 mg/kg is almost completely eliminated after 24 hr (Busch *et al*., 1998). Analysis of tissue distributions in rats that were given IV meloxicam at 1 mg/kg/day for five days and then examined by whole-body autoradiograph showed meloxicam was concentrated at moderate levels in the kidneys, skin, lungs, and intestinal tract and low levels in the skeletal muscle and central nervous system (Busch *et al*., 1998). Buprenorphine when given at 2.4 mg /kg by IV injection showed a half-life of 2.9 hr and almost complete elimination after 24 hr (Yu, Yue, Cui, & Gong, 2006). Since buprenorphine and meloxicam after IV administration are eliminated within 24 hr and have half-lives of 2.9 and 6.8 hr, respectively, this could have consequences for the concentration of analgesics in the ovarian medulla.

Tissue ischemia to the medulla and the cortical ovarian regions occurs during transplantation. However, under normal transplantation conditions both regions are

capable of recovering from necrosis, with angiogenesis primarily taking place in superficial regions like the cortex and not in deeper medullary regions. Analgesics have been shown to alter angiogenesis and vasculogenesis; consequently analgesics can have a negative impact on ovarian transplantation. Meloxicam and buprenorphine have been shown to effect neovascularization, and receptors for buprenorphine are present in ovarian neuronal tissue. Still, the previously reported literature has been conflicting and is inconsistent with this research. The findings of this study indicate that meloxicam and buprenorphine treatments did not promote or inhibit angiogenesis in the medullary region of the ovary post-transplantation in aged recipients under the current administration protocol. Several factors need to be examined to determine the disparity between findings, such as the pharmacokinetics of the analgesics and their ability to reach medullary regions with severed vascular connections.

References

Aghassi, D., Anderson, R., & González, S. (2000). Time-sequence histologic imaging of laser-treated cherry angiomas with *in vivo* confocal microscopy. *Journal of the American Academy of Dermatology, 43*(1), 37-41. doi:10.1067/mjd.2000.105560

Albelda, S. M., Muller, W. A., Buck, C. A., & Newman, P. J. (1991). Molecular and cellular properties of PECAM-1 (endoCAM/CD31): A novel vascular cell-cell adhesion molecule. *The Journal of Cell Biology, 114*(5), 1059-1068. doi:10.1083/jcb.114.5.1059

Asahara, T., Masuda, H., Takahashi, T., Kalka, C., Pastore, C., Silver, M., . . . Isner, J. M. (1999). Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circulation Research, 85*(3), 221-228. doi:10.1161/01.RES.85.3.221

Balasubramanian, S., Ramakrishnan, S., Charboneau, R., Wang, J., Barke, R. A., & Roy, S. (2001). Morphine sulfate inhibits hypoxia-induced vascular endothelial growth factor expression in endothelial cells and cardiac myocytes. *Journal of Molecular and Cellular Cardiology, 33*(12), 2179-2187. doi:10.1006/jmcc.2001.1480

Bamberger, E., & Perrett, C. (2002). Angiogenesis in epithelian ovarian cancer. *Molecular Pathology, 55*(6), 348-359. doi:10.1136/mp.55.6.348

Blaha, M. D., & Leon, L. R. (2008). Effects of indomethacin and buprenorphine analgesia on the postoperative recovery of mice. *Journal of the American Association for Laboratory Animal Science: JAALAS, 47*(4), 8-19. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2694718/

Brodie, T. G. (1903). The perfusion of surviving organs. *The Journal of Physiology, 29*(3), 266-275. doi:10.1113/jphysiol.1903.sp000954

Brown, J. R., & DuBois, R. N. (2005). COX-2: A molecular target for colorectal cancer prevention. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology, 23*(12), 2840-2855. doi:10.1200/JCO.2005.09.051

Busch, U., Schmid, J., Heinzel, G., Schmaus, H., Baierl, J., Huber, C., & Roth, W. (1998). Pharmacokinetics of meloxicam in animals and the relevance to humans. *Drug Metabolism and Disposition: The Biological Fate of Chemicals, 26*(6), 576-584. Retrieved from http://dmd.aspetjournals.org/content/26/6/576.short

Bussolino, F., Mantovani, A., & Persico, G. (1997). Molecular mechanisms of blood vessel formation. *Trends in Biochemical Sciences, 22*(7), 251-256. doi:10.1016/S0968-0004(97)01074-8

Campochiaro, P. A. (2000). Retinal and choroidal neovascularization. *Journal of Cellular Physiology, 184*(3), 301-310. doi:10.1002/1097-4652(200009)

Cha, S. K., Shin, D. H., Kim, B. Y., Yoon, S. Y., Yoon, T. K., Lee, W. S., . . . Lee, D. R. (2014). Effect of human endothelial progenitor cell (EPC)- or mouse vascular endothelial growth factor-derived vessel formation on the survival of Vitrified/Warmed mouse ovarian grafts. *Reproductive Sciences (Thousand Oaks, Calif.), 21*(7), 859-868. doi:1933719113518983

Coffin, J. D., & Poole, T. J. (1988). Embryonic vascular development: Immunohistochemical identification of the origin and subsequent morphogenesis of the major vessel primordia in quail embryos. *Development (Cambridge, England), 102*(4), 735-748. Retrieved from http://dev.biologists.org/content/102/4/735.short

Cohen, P. P. (1945). The metabolism of proteins and amino acids. *Annual Review of Biochemistry, 14*(1), 357-382. doi:10.1146/annurev.bi.14.070145.002041

Cole, D. A., Maxwell, S. E., Arvey, R., & Salas, E. (1993). Multivariate group comparisons of variable systems: MANOVA and structural equation modeling. *Psychological Bulletin, 114*(1), 174. doi:10.1037/0033-2909.114.1.174

Courbiere, B., Caquant, L., Mazoyer, C., Franck, M., Lornage, J., & Salle, B. (2009). Difficulties improving ovarian functional recovery by microvascular transplantation and whole ovary vitrification. *Fertility and Sterility, 91*(6), 2697-2706. doi:10.1016/j.fertnstert.2008.03.012

Cowan, A., Doxey, J., & Harry, E. (1977). The animal pharmacology of buprenorphine, an oripavine analgesic agent. *British Journal of Pharmacology, 60*(4), 547-554. doi:10.1111/j.1476-5381.1977.tb07533.x

Crosby, J. R., Kaminski, W. E., Schatteman, G., Martin, P. J., Raines, E. W., Seifert, R. A., & Bowen-Pope, D. F. (2000). Endothelial cells of hematopoietic origin make a significant contribution to adult blood vessel formation. *Circulation Research, 87*(9), 728-730. doi:10.1161/01.RES.87.9.728

Cursiefen, C., Kuchle, M., & Naumann, G. O. (1998). Angiogenesis in corneal diseases: Histopathologic evaluation of 254 human corneal buttons with neovascularization. *Cornea, 17*(6), 611-613. doi:00003226-199811000-00008

D'Albora, H., Anesetti, G., Lombide, P., Dees, W. L., & Ojeda, S. R. (2002). Intrinsic neurons in the mammalian ovary. *Microscopy Research and Technique, 59*(6), 484-489. doi:10.1002/jemt.10231

De Palma, M., & Naldini, L. (2006). Role of haematopoietic cells and endothelial progenitors in tumour angiogenesis. *Biochimica Et Biophysica Acta (BBA)-Reviews on Cancer, 1766*(1), 159-166. doi:10.1016/j.bbcan.2006.06.003

DeLisser, H. M., Baldwin, H. S., & Albelda, S. M. (1997). Platelet endothelial cell adhesion molecule 1 (PECAM-1/CD31): A multifunctional vascular cell adhesion molecule. *Trends in Cardiovascular Medicine, 7*(6), 203-210. doi:10.1016/S1050-1738(97)00049-2

Dissen, G. A., Lara, H. E., Fahrenbach, W. H., Costa, M. E., & Ojeda, S. R. (1994). Immature rat ovaries become revascularized rapidly after autotransplantation and show a gonadotropin-dependent increase in angiogenic factor gene expression. *Endocrinology, 134*(3), 1146-1154. doi:10.1210/endo.134.3.8119153

Drake, C. J., Brandt, S. J., Trusk, T. C., & Little, C. D. (1997). TAL1/SCL is expressed in endothelial progenitor cells/angioblasts and defines a dorsal-to-ventral gradient of vasculogenesis. *Developmental Biology, 192*(1), 17-30. doi:10.1006/dbio.1997.8751

Edson, M. A., Nagaraja, A. K., & Matzuk, M. M. (2009). The mammalian ovary from genesis to revelation. *Endocrine Reviews, 30*(6), 624-712. doi:10.1210/er.2009-0012

Eklund, L., Bry, M., & Alitalo, K. (2013). Mouse models for studying angiogenesis and lymphangiogenesis in cancer. *Molecular Oncology, 7*(2), 259-282. doi:10.1016/j.molonc.2013.02.007

Ferkowicz, M. J., & Yoder, M. C. (2005). Blood island formation: Longstanding observations and modern interpretations. *Experimental Hematology, 33*(9), 1041-1047. doi:10.1016/j.exphem.2005.06.006

Ferrara, N., Gerber, H., & LeCouter, J. (2003). The biology of VEGF and its receptors. *Nature Medicine, 9*(6), 669-676. doi:10.1038/nm0603-669

Flecknell, P. (2009). *Laboratory animal anaesthesia* Academic Press.

Folkman, J. (2003). Fundamental concepts of the angiogenic process. *Current Molecular Medicine, 3*(7), 643-651. doi:10.2174/1566524033479465

Folkman, J., & Klagsbrun, M. (1987). Angiogenic factors. *Science, 235*(4787), 442-447. doi:10.1126/science.2432664

Fortune, J., Cushman, R., Wahl, C., & Kito, S. (2000). The primordial to primary follicle transition. *Molecular and Cellular Endocrinology, 163*(1), 53-60. doi:10.1016/S0303-7207(99)00240-3

Fuller, J. L., & Sjursen Jr, F. H. (1967). Audiogenic seizures in eleven mouse strains. *Journal of Heredity,* Retrieved from http://psycnet.apa.org/psycinfo/1967-16369-001

Gargiulo, S., Greco, A., Gramanzini, M., Esposito, S., Affuso, A., Brunetti, A., & Vesce, G. (2012). Mice anesthesia, analgesia, and care, part II: Anesthetic considerations in preclinical imaging studies. *ILAR Journal / National Research Council, Institute of Laboratory Animal Resources, 53*(1), E70-81. doi:10.1093/ilar.53.1.70

Garrett, E. R., & Chandran, V. R. (1990). Pharmacokinetics of morphine and its surrogates x: Analyses and pharmacokinetics of buprenorphine in dogs. *Biopharmaceutics & Drug Disposition, 11*(4), 311-350. doi:10.1002/bdd.2510110403

Ginther, O. J., Dierschke, D. J., Walsh, S. W., & Del Campo, C. H. (1974). Anatomy of arteries and veins of uterus and ovaries in rhesus monkeys. *Biology of Reproduction, 11*(2), 205-219. doi:10.1095/biolreprod11.2.205

Gourishankar, S., & Halloran, P. F. (2002). Late deterioration of organ transplants: A problem in injury and homeostasis. *Current Opinion in Immunology, 14*(5), 576-583. doi:10.1016/S0952-7915(02)00386-2

Gupta, K., Kshirsagar, S., Chang, L., Schwartz, R., Law, P. Y., Yee, D., & Hebbel, R. P. (2002). Morphine stimulates angiogenesis by activating proangiogenic and survival-promoting signaling and promotes breast tumor growth. *Cancer Research, 62*(15), 4491-4498. Retrieved from http://cancerres.aacrjournals.org/content/62/15/4491.short

Hamberg, M., Svensson, J., & Samuelsson, B. (1975). Thromboxanes: A new group of biologically active compounds derived from prostaglandin endoperoxides. *Proceedings of the National Academy of Sciences of the United States of America, 72*(8), 2994-2998. Retrieved from http://www.pnas.org/content/72/8/2994.abstract

Horak, E., Klenk, N., Leek, R., LeJeune, S., Smith, K., Stuart, N., . . . Stepniewska, K. (1992). Angiogenesis, assessed by platelet/endothelial cell adhesion molecule antibodies, as indicator of node metastases and survival in breast cancer. *The Lancet, 340*(8828), 1120-1124. doi:10.1016/0140-6736(92)93150-L

Israely, T., Dafni, H., Granot, D., Nevo, N., Tsafriri, A., & Neeman, M. (2003). Vascular remodeling and angiogenesis in ectopic ovarian transplants: A crucial role of pericytes and vascular smooth muscle cells in maintenance of ovarian grafts. *Biology of Reproduction, 68*(6), 2055-2064. doi:10.1095/biolreprod.102.011734

Johnson, R. E., Fudala, P. J., & Payne, R. (2005). Buprenorphine: Considerations for pain management. *Journal of Pain and Symptom Management, 29*(3), 297-326. doi:10.1016/j.jpainsymman.2004.07.005

Kang, D. H., Joly, A. H., Oh, S. W., Hugo, C., Kerjaschki, D., Gordon, K. L., . . . Johnson, R. J. (2001). Impaired angiogenesis in the remnant kidney model: I. potential role of vascular endothelial growth factor and thrombospondin-1. *Journal of the American Society of Nephrology: JASN, 12*(7), 1434-1447. Retrieved from http://jasn.asnjournals.org/content/12/7/1434.short

Katzung, B. G., Masters, S. B., & Trevor, A. J. (2009). *Basic & Clinical Pharmacology*, (11ed). New York: McGraw-Hill Medical.

Kikuta, A., Macchiarelli, G., & Murakami, T. (1991). Microvasculature of the ovary. *Ultrastructure of the ovary* (pp. 239-254) Springer. Retrieved from http://link.springer.com/chapter/10.1007/978-1-4615-3944-5_15#page-1

Kim, S. S., Soules, M. R., & Battaglia, D. E. (2002). Follicular development, ovulation, and corpus luteum formation in cryopreserved human ovarian tissue after xenotransplantation. *Fertility and Sterility, 78*(1), 77-82. doi:10.1016/S0015-0282(02)03144-8

Kim, S. S., Yang, H. W., Kang, H. G., Lee, H. H., Lee, H. C., Ko, D. S., & Gosden, R. G. (2004). Quantitative assessment of ischemic tissue damage in ovarian cortical tissue with or without antioxidant (ascorbic acid) treatment. *Fertility and Sterility, 82*(3), 679-685. doi:10.1016/j.fertnstert.2004.05.022

Kim, S., Kim, J., Papadopoulos, J., Wook Kim, S., Maya, M., Zhang, F., . . . Fidler, I. J. (2009). Circulating monocytes expressing CD31: Implications for acute and chronic angiogenesis. *The American Journal of Pathology, 174*(5), 1972-1980. doi:10.2353/ajpath.2009.080819

Kohn, D. F., Martin, T. E., Foley, P. L., Morris, T. H., Swindle, M. M., Vogler, G. A., & Wixson, S. K. (2007). Guidelines for the assessment and management of pain in rodents and rabbits. *Journal of the American Association for Laboratory Animal Science, 46*(2), 97-108. Retrieved from http://www.ingentaconnect.com/content/aalas/jaalas/2007/00000046/00000002/art00016

Koodie, L., Ramakrishnan, S., & Roy, S. (2010). Morphine suppresses tumor angiogenesis through a HIF-1α/p38MAPK pathway. *The American Journal of Pathology, 177*(2), 984-997. doi:10.2353/ajpath.2010.090621

Le, A. H., Bonachea, L. A., & Cargill, S. L. (2014). Meloxicam and buprenorphine treatment after ovarian transplantation does not affect estrous cyclicity and follicular integrity in aged CBA/J mice. *PloS One, 9*(8), e106013. doi:10.1371/journal.pone.0106013

Leahy, K. M., Ornberg, R. L., Wang, Y., Zweifel, B. S., Koki, A. T., & Masferrer, J. L. (2002). Cyclooxygenase-2 inhibition by celecoxib reduces proliferation and induces apoptosis in

angiogenic endothelial cells in vivo. *Cancer Research, 62*(3), 625-631. Retrieved from http://cancerres.aacrjournals.org/content/62/3/625.short

Li, B., Sharpe, E. E., Maupin, A. B., Teleron, A. A., Pyle, A. L., Carmeliet, P., & Young, P. P. (2006). VEGF and PIGF promote adult vasculogenesis by enhancing EPC recruitment and vessel formation at the site of tumor neovascularization. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology, 20*(9), 1495-1497. doi:fj.05-5137fje

Lutfy, K., Eitan, S., Bryant, C. D., Yang, Y. C., Saliminejad, N., Walwyn, W., . . . Evans, C. J. (2003). Buprenorphine-induced antinociception is mediated by mu-opioid receptors and compromised by concomitant activation of opioid receptor-like receptors. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 23*(32), 10331-10337. doi:23/32/10331

Marzouk, K., Lawen, J., Alwayn, I., & Kiberd, B. A. (2013). The impact of vascular anastomosis time on early kidney transplant outcomes. *Transplantation Research, 2*(8) doi:10.1186/2047-1440-2-8

Mason, J. B., Cargill, S. L., Anderson, G. B., & Carey, J. R. (2009). Transplantation of young ovaries to old mice increased life span in transplant recipients. *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences, 64*(12), 1207-1211. doi:10.1093/gerona/glp134

McDonald, D. M., & Choyke, P. L. (2003). Imaging of angiogenesis: From microscope to clinic. *Nature Medicine, 9*(6), 713-725. doi:10.1038/nm0603-713

Murray, P. D. F. (1932). The development in vitro of the blood of the early chick embryo. *Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character*, 497-521. Retrieved from http://www.jstor.org/stable/81556

Nalini Sehgal, M., Howard Smith, M., & Laxmaiah Manchikanti, M. (2011). Peripherally acting opioids and clinical implications for pain control. *Pain Physician, 14*, 249-258. Retrieved from http://www.painphysicianjournal.com/2011/may/2011;14;249-258.pdf

Noden, D. M. (1990). Origins and Assembly of Avian Embryonic Blood Vesselsa. *Annals of the New York Academy of Sciences*, *588*(1), 236-249. doi:10.1111/j.1749-6632.1990.tb13214.x

Oliveira, T. M., Sakai, V. T., Machado, M. A. A., Dionísio, T. J., Cestari, T. M., Taga, R., . . . Santos, C. F. (2008). COX-2 inhibition decreases VEGF expression and alveolar bone loss during the progression of experimental periodontitis in rats. *Journal of Periodontology, 79*(6), 1062-1069. doi:10.1902/jop.2008.070411

Plumb, D. C. (2005). *Plumb's veterinary drug handbook.* PharmaVet.

Poonawala, T., Levay-Young, B. K., Hebbel, R. P., & Gupta, K. (2005). Opioids heal ischemic wounds in the rat. *Wound Repair and Regeneration, 13*(2), 165-174. doi:10.1111/j.1067-1927.2005.130207.x

Ricciotti, E., & FitzGerald, G. A. (2011). Prostaglandins and inflammation. *Arteriosclerosis, Thrombosis, and Vascular Biology, 31*(5), 986-1000. doi:10.1161/ATVBAHA.110.207449

Risau, W. (1997). Mechanisms of angiogenesis. *Nature, 386*(6626), 671-674. doi:10.1038/386671a0

Risau, W., & Flamme, I. (1995). Vasculogenesis. *Annual review of cell and developmental biology*, *11*(1), 73-91. doi: 10.1146/annurev.cb.11.110195.000445

Russell, R. B., Breed, J., & Barton, G. J. (1992). Conservation analysis and structure prediction of the SH2 family of phosphotyrosine binding domains. *FEBS Letters, 304*(1), 15-20. doi:10.1016/0014-5793(92)80579-6

Sabin, F. R. (1920). Studies on the origin of blood-vessels and of red blood-corpuscles as seen in the living blastoderm of chicks during the second day of incubation. *Contributions to Embryology,* 213-262. Retrieved from: http://www.worldcat.org/title/studies-on-the-origin-of-blood-vessels-and-of-red-blood-corpuscles-as-seen-in-the-living-blastoderm-of-chicks-during-the-second-day-of-incubation/oclc/15557247

Shweiki, D., Itin, A., Neufeld, G., Gitaygoren, H., & Keshet, E. (1993). Patterns of expression of vascular endothelial growth-factor (vegf) and vegf receptors in mice suggest a role in hormonally regulated angiogenesis. *Journal of Clinical Investigation, 91*(5), 2235-2243. doi:10.1172/JCI116450

Starlz, T. E., Kaupp, H. A.,Jr, Brock, D. R., Lazarus, R. E., & Johnson, R. V. (1960). Reconstructive problems in canine liver homotransplantation with special reference to the postoperative role of hepatic venous flow. *Surgery, Gynecology & Obstetrics, 111*, 733-743. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2729187/

Su, R., Sun, C., & Pham, T. D. (2012). Junction detection for linear structures based on Hessian, correlation and shape information. *Pattern Recognition*, *45*(10), 3695-3706. doi: 10.1016/j.patcog.2012.04.013

Vane, J. (1994). Towards a better aspirin. *Nature, 367*(6460), 215-216. doi:10.1038/367215a0

Vokes, S. A., & Krieg, P. A. (2002). Endoderm is required for vascular endothelial tube formation, but not for angioblast specification. *Development (Cambridge, England), 129*(3), 775-785. Retrieved from http://dev.biologists.org/content/129/3/775.short

Webb, R. H. (1996). Confocal optical microscopy. *Reports on Progress in Physics, 59*(3), 427. doi:10.1088/0034-4885/59/3/003

Weissman, A., Gotlieb, L., Colgan, T., Jurisicova, A., Greenblatt, E. M., & Casper, R. F. (1999). Preliminary experience with subcutaneous human ovarian cortex transplantation in the NOD-SCID mouse. *Biology of Reproduction, 60*(6), 1462-1467. doi:10.1095/biolreprod60.6.1462

Whitten, W. K. (1956). Modification of the oestrous cycle of the mouse by external stimuli associated with the male. *The Journal of Endocrinology, 13*(4), 399-404. doi:10.1677/joe.0.0130399

Willoughby, D. A., Moore, A. R., & Colville-Nash, P. R. (2000). COX-1, COX-2, and COX-3 and the future treatment of chronic inflammatory disease. *The Lancet, 355*(9204), 646-648. doi:10.1016/S0140-6736(99)12031-2

Wittert, G., Hope, P., & Pyle, D. (1996). Tissue distribution of opioid receptor gene expression in the rat. *Biochemical and Biophysical Research Communications, 218*(3), 877-881. doi:10.1006/bbrc.1996.0156

Yu, G., Yue, Y. J., Cui, M. X., & Gong, Z. H. (2006). Thienorphine is a potent long-acting partial opioid agonist: A comparative study with buprenorphine. *The Journal of Pharmacology and Experimental Therapeutics, 318*(1), 282-287. doi:jpet.105.099937